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# Sodium/proton exchanger 3 (NHE3) and sudden infant death syndrome (SIDS)

Jacqueline Studer · Christine Bartsch · Cordula Haas

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**Abstract** The sodium/proton exchanger protein 3 (NHE3) is located in chemosensitive areas of the medulla oblongata and plays an important role in the central control of respiration. Overexpression of NHE3 is correlated with lower respiration and might therefore contribute to the vulnerability of infants dying suddenly and unexpected (sudden infant death syndrome, SIDS). Our aim in this study was to verify already reported genetic variations in the NHE3 gene in an independent SIDS cohort from Switzerland. Two single nucleotide polymorphisms (SNPs) in the promoter region (G1131A and C1197T) and one variation in the coding sequence of exon 16 (C2405T) in the NHE3 gene were analyzed in 160 Caucasian SIDS infants and 192 Swiss adult controls by using a single base extension method (SNaPshot multiplex). No significant differences were detected in the allelic frequencies of the three NHE3 polymorphisms between SIDS cases and controls. We conclude that the three investigated NHE3 SNPs are unlikely to play a major role in the pathogenesis of SIDS in Caucasian infants. However, further genetic investigations in different ethnicities are required to determine whether variations in NHE3 are associated with an increased SIDS risk.

**Keywords** Sudden infant death syndrome · Genetic risk factors · Sodium/proton exchanger 3 · Single nucleotide polymorphism · Single base extension

## Introduction

Sudden infant death syndrome (SIDS) is defined as the sudden and unexpected death of an apparently healthy infant younger

than 1 year of age [1]. The identification and prevention of several environmental risk factors, such as the prone sleeping position, sharing bed with parents, or exposure to smoking, have contributed to a drastic reduction of SIDS cases in the last 20 years [2–4]; however, SIDS is still the leading cause of postneonatal infant death in developed countries [5]. Today, the pathogenesis of SIDS is widely accepted as a multifactorial disorder that involves a critical developmental period in combination with environmental and genetic risk factors [6]. The most important genetic risk factors with respect to SIDS are variants in genes leading to a dysfunctional immune system response, defects in the autonomic regulation of cardiovascular activities, and respiratory failures [7–9].

The control of autonomic respiration is located in the medulla oblongata where a change in  $\text{CO}_2/\text{H}^+$  is sensed by specialized central chemosensitive neurons [10]. Elevation of  $\text{CO}_2/\text{H}^+$  levels increases the firing rate of these brainstem neurons, which is mediated by an increase of intracellular free protons and increased respiration. To maintain the acid-base balance, an electroneutral exchange of  $\text{Na}^+$  for  $\text{H}^+$  is accomplished by transmembrane sodium/proton exchangers (NHE) [11]. NHEs exist in at least nine different isoforms in humans, whereas all isoforms have tissue-specific distributions [12]. Inhibition of the subtype sodium/proton exchanger 3 (NHE3, also known as SLC9A3) resulted in a lower apneic threshold and an increased respiration in rabbits, demonstrating that this NHE isoform is involved in the central respiratory response [13]. Additionally, the level of NHE3 mRNA was found to be inversely correlated to the degree of respiratory drive, meaning high ventilation is correlated with low NHE3 expression levels and vice versa [14]. It was supposed that an overexpression of NHE3 leading to lower ventilation may predispose individuals to central apnea and sleep-disordered breathing and might therefore also contribute to the vulnerability of SIDS infants [15]. Genetic investigations of the NHE3 gene in German SIDS infants revealed three variants that were

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significantly more frequent in SIDS infants than in control subjects [16]. Two of these single nucleotide polymorphisms (SNPs) are located in the promoter region of the NHE3 gene (G1131A and C1197T) and the third SNP is located on exon 16 (C2405T). Since these findings have not been replicated in an independent SIDS cohort, our aims were to investigate the three NHE3 polymorphisms in a SIDS cohort from Switzerland and to verify these variants as possible predisposing factors in the occurrence of SIDS.

## Material and methods

### Study population

Our study population was composed of 160 SIDS cases and initially 532 adult controls. All SIDS cases were collected between 1985 and 2012 at the Institute of Legal Medicine in Zurich, Switzerland, whereas most of them were examined by the same forensic pathologist. Since 2004, SIDS cases were classified according to the San Diego definition, which includes a complete autopsy, review of the circumstances of death, and examination of the clinical history [1]. 40 (23 males/17 females) SIDS cases out of the 160 infants were determined as genuine SIDS cases belonging to category I with a normal clinical history and no assigned cause of death. The remaining 120 (72 males/48 females) SIDS infants were categorized in category II due to slight infections prior to death, preterm birth, or other deviations to category I requirements. According to the autopsy records, all SIDS infants were Caucasian and most of them had parents with Swiss origin. The total SIDS cohort was composed of 95 males and 65 females and the median age was  $14.9 \pm 10.4$  weeks (range 0.6–48.1 weeks). Information about environmental risk factors such as the prone sleeping position or exposure to parental smoking behavior was available from the autopsy reports of most infants.

The initial control population was composed of 532 healthy Caucasian adults (228 females/304 males). However, genetic investigations of the three NHE3 SNPs showed that the allelic distribution of these SNPs deviated from the Hardy-Weinberg equilibrium (HWE). Assuming that this may be an indicator for population heterogeneity [17], the statistical analysis was only performed with those adults that reported Swiss descent ( $n=192$ ; 77 females/115 males).

Ethical approval for this study was provided by the local ethics committee in Zurich (KEK-ZH-Nr. 2013-0086), and the study was conducted in full conformance with Swiss laws and regulations.

### DNA extraction and quantification

Genomic DNA of SIDS infants was obtained from tissues stored in alcohol or from alcohol-fixed paraffin-embedded tissue blocks [18]. Most of the tissues were kidney or tongue (otherwise heart, muscle, or brain) because of reported good postmortem DNA stability in these tissues [19]. DNA extractions were performed using the QIAamp DNA Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol. DNA of the adult controls was extracted from blood [20] or buccal swabs [21]. All DNA quantities were determined with the Quantifiler<sup>TM</sup> Human DNA quantification kit (Life Technologies, Rotkreuz, Switzerland).

### SNP analysis

Multiplex PCR amplification was performed with 1 ng DNA as a template using the QIAGEN Multiplex PCR Kit (Qiagen) according to the manufacturer's protocol in a total volume of 15  $\mu$ l and with the same PCR primer sequences as described by Poetsch et al. [16]. Primer concentrations were 0.1  $\mu$ M for C2405T, 0.05  $\mu$ M for G1131A, and 0.1  $\mu$ M for C1197T. Thermal cycling conditions were as follows: initial incubation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 57–64 °C for 90 s (increase of 0.2 °C per cycle), 72 °C for 60 s, and a final extension at 72 °C for 60 min. The three SNP variations (C2405T, G1131A, and C1197T) were then genotyped in a multiplex reaction by using a single base extension (SBE) method with the SNaPshot multiplex kit (Life Technologies) according to the manufacturer's protocol. SBE primer sequences were the same as described by Poetsch et al. [16]. Primer concentrations were 1  $\mu$ M for C2405T, 0.05  $\mu$ M for G1131A, and 0.2  $\mu$ M for C1197T. Thermal cycling conditions were as follows: rapid thermal ramp to 96 °C followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. SBE products were detected by capillary electrophoresis on a 3130xl Genetic Analyzer (Life Technologies) and analyzed with the GeneMapper ID-X 1.2 software (Life Technologies). Inconclusive results were repeated at least once.

### Statistical analysis

Data analysis was performed with the statistical software IBM SPSS version 20 (SPSS Inc., Chicago, USA). The HWE was calculated with a web-based HWE calculator software [22]. A  $\chi^2$  value of  $\geq 3.84$  indicated a deviation of the HWE at a significance level of 0.05. Differences of the genetic investigations between SIDS and controls were compared by a Pearson  $\chi^2$  test in a  $2 \times 2$  table for allelic frequencies and in a  $3 \times 2$  table for genotypes. A  $p$  value  $\leq 0.05$  was considered statistically significant. All reported  $p$  values are two-sided.

## Results and discussion

In our study population of 160 Caucasian SIDS cases and 192 Swiss adult controls, we found no differences in the allelic distribution of the three investigated NHE3 SNPs C2405T, G1131A, and C1197T between SIDS cases and controls (C2405T:  $p$  value=0.376; G1131A:  $p$  value=0.578; C1197T:  $p$  value=0.460) (Table 1). The homozygous TT genotype of C2405T and the homozygous AA genotype of G1131A were found only rarely in SIDS cases (1.5 and 1.9 %, respectively) and controls (1.0 and 1.6 %, respectively). The homozygous TT genotype of C1197T was neither found in SIDS cases nor in the control population. HWE expectations were met for all three SNPs (C2405T, G1131A, and C1197T) in the SIDS group and the Swiss controls. SIDS group I ( $n=40$ ) and SIDS group II ( $n=120$ ) were separately compared to the control population, but no significant differences in the allelic distribution of the three NHE3 polymorphisms were detected in the two SIDS groups (data not shown).

Furthermore, the allelic distributions were similar between female and male SIDS infants and between infants that died in the months of highest SIDS risk (2–4 months) versus all other months (data not shown). Infants found dead in the prone sleeping position were further compared to infants in the non-prone sleeping position, as sleeping on the stomach might be an external trigger for decreased breathing drive. Information on sleeping position of SIDS infants was available in 94 cases, 64 cases of which were found dead in the prone sleeping position. However, the allelic frequencies of C2405T, G1131A, and C1197T were similarly distributed in infants found dead in the prone position versus the non-prone position (data not shown).

The C2405T is located on exon 16 and results in an amino acid change from arginine (Arg) to cysteine (Cys) in the distal region of the COOH terminal. This domain is regulated by the binding of phosphatase 2A (PPA2), NHE regulating factor 1 (NHERF1), and megalin (LRP2), which were shown to inhibit NHE3 expression [23, 24]. Poetsch et al. hypothesized that a change in the amino acid structure at this position may prevent

the binding of such expression regulating factors and thus leading to an overexpression of NHE3 [16]. The two other variations, G1131A and C1197T, are both located in the promoter region where they may alter the promoter activity of NHE3. In our study population, the homozygous TT genotype of C2405T was found in 1.5 % of all SIDS cases, the homozygous AA genotype of G1131A in 1.9 % of all SIDS cases, and the homozygous TT genotype of C1197T was not observed in any SIDS case. Therefore, it seems rather unlikely that these variations play a pathogenic role in the occurrence of SIDS in Caucasian infants.

Our results are in contrast to the previously published findings in 251 German SIDS infants and 220 controls, which were composed of 170 adult controls and 50 infant controls younger than 1 year of age [16]. We believe that infant controls less than 1 year of age do not represent an optimal control population, as the occurrence of SIDS cannot be excluded in those infants. Under the assumption that SNP genotypes are not affected by age, controls should be gender- but not age-matched to the SIDS cohort [25].

This is our fourth study trying to verify previously described genetic risk factors in an independent SIDS population from Switzerland. As in this study, we could not confirm significant associations between SIDS and polymorphisms in the regulatory region of the serotonin transporter (5-HTT) gene [26–28], in the tyrosine hydroxylase marker (TH01) [29, 30], (Studer J., Bartsch C., and Haas C. Tyrosine hydroxylase TH01 9.3 allele in the occurrence of sudden infant death syndrome (SIDS) in Swiss Caucasians, accepted for publication) and in the aquaporin-4 (AQP4) gene [31]. Such inconsistent results were also reported in other SIDS association studies, e.g., in regard to polymorphisms in the monoamine oxidase A (MAOA) or in serotonin (5-HTT) pathway genes [32–37], (Studer J., Bartsch C., and Haas C. Aquaporin-4 polymorphisms and brain/body weight ratio in sudden infant death syndrome (SIDS) accepted for publication). A meta-analysis of 370 association studies demonstrated that first studies often suggest a stronger genetic effect than is found by subsequent studies [38], which might be

**Table 1** Genotypes and allelic distributions of NHE3 SNPs in SIDS infants compared to controls

	Genotypes [Number (%)]			Alleles [Number (%)]		$p$ value
C2405T	CC	CT	TT	C	T	
SIDS (n=160)	118 (73.5)	40 (25)	2 (1.5)	276 (86.2)	44 (13.8)	0.376
Controls (n=192)	132 (68.8)	58 (30.2)	2 (1.0)	322 (83.9)	62 (16.1)	
G1131A	GG	GA	AA	G	A	
SIDS (n=160)	113 (70.6)	44 (27.5)	3 (1.9)	270 (84.4)	50 (15.6)	0.578
Controls (n=192)	129 (67.2)	60 (31.2)	3 (1.6)	318 (82.8)	66 (17.2)	
C1197T	CC	CT	TT	C	T	
SIDS (n=160)	158 (98.8)	2 (1.2)	0 (0)	318 (99.4)	2 (0.6)	0.460
Controls (n=192)	191 (99.5)	1 (0.2)	0 (0)	383 (99.7)	1 (0.3)	

one possible explanation for the conflicting results of these replication studies. It is further recommended to perform HWE tests in association studies, as significant differences between the observed and expected genotype frequencies under HWE may indicate genotyping errors or population stratification and can inflate the chance of a false-positive association [39, 40]. Even small amounts of population admixture can shift the results toward an association, and therefore, it is important to have a well-defined control population in regard to ethnic and geographic background [41]. Additionally, the well-known genetic heterogeneity of SIDS cases and small sample sizes can contribute to the different outcomes in SIDS association studies.

## Conclusion

Although we could not confirm that the three NHE3 polymorphisms may be predisposing risk factors in the occurrence of SIDS in Caucasian infants, NHE3 still remains of interest due to its involvement in determining the set point of normal breathing. Therefore, further genetic investigations of the NHE3 promoter region and coding sequences should be performed, also in other ethnical SIDS cohorts. Additionally, gene expression studies are required to confirm the hypothesis that the NHE3 mRNA is overexpressed in the brainstem of SIDS infants.

**Conflict of interest** The authors declare that they have no conflict of interest.

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